The pathways of blood coagulation

27A

The pathways of blood coagulation

DOOR DR. H. C. HEMKER

Folia Medica Neerlandica - Vol. 10/4-5, 1967.

In 1963 MACFARLANE published a theory on the reaction mechanism of blood coagulation known as the *cascade hypothesis*[35]. A short time later, DAVIES and RATNOFF made a similar suggestion[8]. The cascade hypothesis can be viewed as a synthesis of the knowledge in the field of the biochemistry of blood coagulation* at that moment and as a unifying theory of the interaction of blood clotting enzymes. The experimental evidence on which this theory was based has been presented in various articles[36,37]. There is little doubt that along general lines this theory presents the best over-all view of present ideas on the blood coagulation reaction scheme. Within its broad structure, however, a considerable amount of detailed investigation remains to be done.

In the present review I will first state the cascade theory and then discuss some recent elaborations and modifications of it. The theory contains sufficient information on coagulation chemistry for all purposes except specialized research.

The basis of the cascade theory is the concept of sequential activation. This concept is perhaps best explained in terms of an example using well-known enzymes. The proteolytic enzyme chymotrypsin (E.C. 3.4.4.5,where E.C. stands for Enzyme Code[17]) is known to originate from the inactive zymogen chymotrypsinogen. The activation of chymotrypsinogen occurs by splitting of the chymotrypsinogen molecule into two parts. One of these parts is the active chymotrypsin molecule and the other is a peptide which, as long as it was part of the zymogen, had been masking the active site of the chymotrypsin pre-existent in the chymotrypsinogen. This reaction is represented in Fig. 1.

Now, the splitting of the protein molecule chymotrypsinogen into an active and an inactive part is, in itself, a proteolytic action. So we need not be surprised that a proteolytic enzyme like trypsin (E.C.3.4.4.4) can bring about this activation. Trypsin, however, originates from trypsinogen in a manner analogous to the way in which chymotrypsin originates from chymotrypsinogen. Still another proteolytic enzyme, enteropeptidase (E.C.3.4.4.8) can convert trypsinogen into trypsin.

Consequently, the addition of a few molecules of enteropeptidase to a mixture of trypsinogen and chymotrypsinogen would be able to cause the gradual appearance of trypsin, and since the tryp-

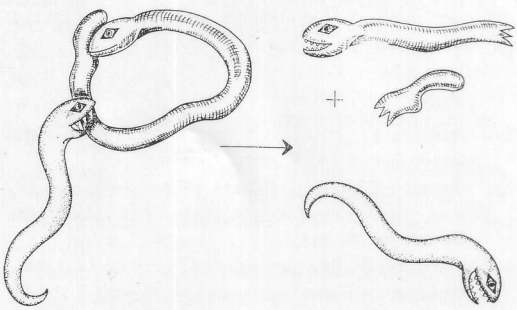

Fig. 1. A proteolytic enzyme converting a zymogen into an active enzyme.

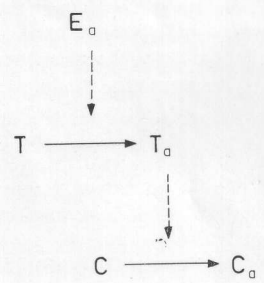

Fig. 2. A simple enzyme cascade.

From the Haematology Section of the Department of Internal Medicine, University Hospital, Leiden.

Manuscript received April 1967.

sin would act on the chymotrypsinogen, chymotrypsin would arise in the mixture more and more rapidly.

For the sake of argument this situation has been simplified somewhat, but in general this course of events is perfectly feasible[7]. If we denote the zymogen by its initial, and the active enzyme derived from the zymogen by the same letter with the subscript a, the reaction sequence just described can be represented as in Fig. 2. This example demonstrates two facts: In the first place, sequential activation is possible in well-defined biochemical systems; and in the second place, sequential activation has an amplifying effect upon the rate of appearance of the final product. This latter point will not be explained at length here because it is easily seen intuitively; a theoretical treatment of the kinetics involved has been given elsewhere[19,31].

The cascade theory applies the concept of sequential activation to the blood-coagulation zymogens and enzymes, or blood-clotting factors as they are usually called. A clotting factor is defined as an entity lacking in the plasma of patients with certain circumscript disorders of their clotting mechanism. In order to permit the recognition of a new clotting factor, a coagulation defect must differ from the defects caused by a deficiency of previously described clotting factors. The disorder of two patients is, by definition, unequal when their plasmas, after mixing, show correction of the coagulation tests by which the plasmas have been judged abnormal and equal when such a correction does not take place. Furthermore, all known coagulation-factor-deficiencies appear to be caused by different defects in genetic material as judged from the way in which these illnesses are inherited[60].

Most factors have never been prepared in a sufficiently pure state in the quantities necessary to determine enough chemical or physico-chemical properties to define them unambiguously in this way. Nevertheless, for several clotting factors the molecular weight, amino-acid composition, electrophoretic mobility, etc. have been determined. If nothing else, these results show that clotting factors are indeed plasma proteins. After the extraordinary progress made in biochemical genetics in recent years, it may be questioned whether a genetical definition, which the clotting factors *do* have, does not provide just as good a basis for the recognition of a protein as would physico-chemical determinations – which are still largely lacking for the clotting factors.

Because an enzyme is defined as a protein with catalytic properties[10] and because the activated clotting factors do indeed have catalytic properties since they catalyse the formation of thrombin from prothrombin, clotting factors fit perfectly into the definition of enzymes. Their inactive precursors would then be zymogens. Yet even extremely competent enzymologists who are not especially familiar with the field of blood clotting hesitate to recognize them as such[10] and consequently the blood-clotting enzymes (except thrombin E.C.3.4.4.13) are not mentioned in the list of known enzymes published by the Commission on Enzymes[17].

The International Committee on Hemostasis and Thrombosis solved the ambiguities in the nomenclature of bloodclotting factors by assigning roman numerals to the factors whose existence has been well established. A list of these numbers, together with current synonyms, is given in Table I.

Put briefly, the cascade theory of MACFARLANE depicts the interaction of these factors as follows: Contact with a foreign surface converts Factor XII into an active enzyme, denoted as Factor XII_a. This enzyme then activates Factor XI, Factor XI_a converts Factor IX, and so on, according to the scheme shown in Fig. 3, until finally thrombin produces fibrin from fibrinogen.

TABLE I: NOMENCLATURE OF BLOOD-CLOTTING FACTORS

factor	*name*
I	Fibrinogen
II	Prothrombin (thrombin is II_a)
III	Tissue factor
IV	Ca^{++}-ion
V	Pro-accelerin
VI	Accelerin, identical to V_a
VII	Proconvertin
VIII	Antihaemophilic factor A
IX	Christmas factor, antihaemophilic factor B
X	Stuart-Prower factor
XI	PTA, plasma thromboplastin antecedent
XII	HF, Hageman factor
XIII	FSF, fibrin-stabilizing factor
?	Tatsumi factor

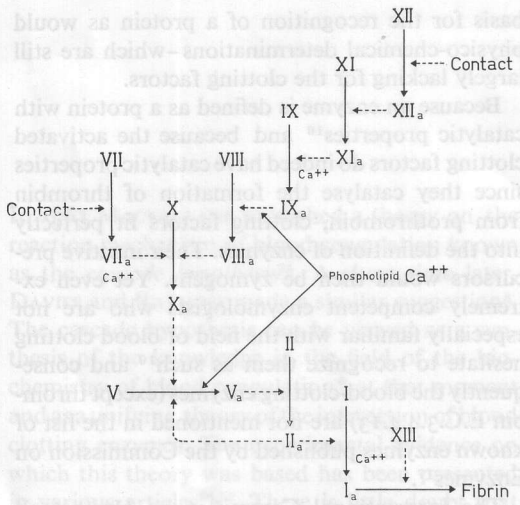

Fig. 3. The clotting cascade.

The clotting time of normal decalcified plasma after recalcification (recalcification time) is of the order of magnitude of several minutes, say 240 seconds. After addition of a tissue extract, such as can be derived from the brain, lung, placenta, or other organs, this time decreases to about 15 seconds (the time thus obtained is called prothrombin time). Plasma deficient in one of the Factors XII, XI, IX, or VIII has a considerably longer recalcification time than normal plasma, but the prothrombin time is equal to that of normal plasma. On the other hand, Factor VII-deficient plasma has a recalcification time that is about normal but a prothrombin time as long as its recalcification time. In Factor I, II, V, and X deficiencies both recalcification time and prothrombin time are prolonged as compared to normal plasma. These findings are sufficiently explained if we assume that an entity in the tissue extract together with Factor VII has the same action as the combined Factors XII, XI, IX, and VIII. In the cascade scheme this is rendered by a branching at the level of Factor X. Because the pathway of X activation via tissue factor and Factor VII requires the presence of a "tissue factor" extraneous to normal plasma, it is called the "*extrinsic pathway*"[9].

The first reaction of the intrinsic pathway has been demonstrated most elegantly by VROMAN[33, 62], who was able to demonstrate by the use of an ellipsometer* that Factor XII attaches to certain surfaces and after adsorption is removed from the surface by Factor XI. This implies that upon absorption on these surfaces Factor XII changes its tertiary structure (i.e. the way in which its polypeptide chain is folded), and after this change is capable of interaction with Factor XI. This behaviour explains why blood begins to clot when brought into contact with foreign surfaces. Factor XII has been rather well defined by the work of SCHOENMAKERS *et al.*[47,48,49]. as a sialoglycoprotein with esterase and proteolytic properties. Activated Factor XI also has esterase properties but ones differing from those of Factor XII_a[39]. SCHOENMAKERS *et al.* have also found suggestive evidence that the final active entity arising from the interaction of the Factors XII_a and XI is a complex consisting of both proteins, rather than activated Factor XI arising from Factor XI by enzymic action of activated Factor XII. The final active entity will be called *contact product* here, with negation of the question of its actual composition.

Not many pertinent data are available about Factor IX. Like most other factors it is a plasma protein synthesized by the liver. It is defined as the factor lacking in haemophilia B. Together with Factors II, VII, and X, it can be separated from plasma rather easily, and the product thus obtained corrects plasmas with Factor II, VII, X or IX deficiencies *in vitro* and raises their blood level *in vivo*[4,53,55]. The separation of Factors II, VII, IX, and X from each other has offered considerable difficulties, probably because these factors occur in the form of a molecular complex [2,63]. The fact that these activities can be purified considerably and still stick together in a complex has even led to a theory, most ardently defended by SEEGERS, according to which the entities described by Factors II, VII, IX, and X are in reality one

*An ellipsometer is a device for analysing the properties of elliptically polarized light. When parallel polarized light is reflected on a surface upon which a very thin layer of material is adsorbed, the reflected light will be elliptically polarized. The properties of this elliptically polarized light are determined by the thickness and the refractive index of the layer adsorbed. Thus analysis of the state of polarization of the reflected light yields data that allow determination of the refractive index and thickness of the layer adsorbed (see also ref. 43).

molecule[51]. A close look at the arguments erupting in the coagulation literature for more than 20 years[2,51] shows that the only real difference in opinion is, that SEEGERS assumes that the activities known as Factor II, VII, IX, and X, are bound covalently (i.e. by "normal" chemical bonds) when not activated, whereas most other authors think that the binding, if present, is probably effected by hydrogen bonds or by interaction of hydrophobic parts of the molecules. The fact that a test plasma consisting of equal parts of Factor II- and Factor VII-deficient plasmas immediately after mixture shows virtually normal results of clotting tests, argues strongly in favour of the latter view. Moreover, recent research has shown that the factors under discussion can be separated. In separated form they correct the deficiency of only one kind of deficient plasma. Only after remixing do these separate fractions appear to exhibit the autocalytic properties originally described by SEEGERS as typical of his "pure" preparation[30]. In addition to this, it was found to be possible to obtain these clotting factors in a form several times purer than the best preparations described by SEEGERS[24,45,15]. Nevertheless, the difficulties in this field have resulted in the failure to sufficiently purify and biochemically define Factor IX.

Factor IX is thought to be activated by the contact product. At a recent congress, evidence was put forward by Japanese workers that still another factor, called the *Tatsumi factor*, might have a place in the scheme between Factors XI and IX[3]. The Tatsumi-factor deficiency was found in a Japanese family. The properties of this factor are said to closely resemble those of Factors II, VII, IX, and X, and it is thought to be the same factor as the Thorium vulnerable factor. This may serve to illustrate the fact that postulating new factors is still a popular pastime in coagulation research.

Factor VIII has been purified to a great extent but sufficient physico-chemical information is still lacking[44,58,59]. It is defined as the factor absent in classic haemophilia or haemophilia A.

Factor V has been purified and its molecular weight and some other properties are known[42,14]. It is interesting to note that Factors I (fibrinogen), V, and VIII seem, like Factors II, VII, IX, and X, to form a family. Factors I, V, and VIII are often found together after various isolation procedures. Moreover, a role has been claimed for thrombin in the physiological conversion of Factors V and VIII during the clotting process; in addition to which, of course, thrombin is the natural enzyme acting on Factor I. This is a most interesting point. RAPPAPORT has provided convincing evidence that Factor VIII as it occurs in plasma is incapable of participation in the clotting process[46]. Only after the action of a very small but detectable amount of thrombin is Factor VIII from plasma converted into a form (called $VIII_i$ bij RAPPAPORT) that can take part in the clotting reactions. The subscribed t in the term $VIII_t$ comes from thrombin. I would prefer to have this subscript seen as the t of "transient", which would allow us to use the same subscript in all similar cases. It is known that Factor V is also modified and activated by thrombin[38], by RUSSELS viper venom, and possibly by activated Factor VII[23].

It is possible that Factors I, V and VIII are found together in most purification procedures because a Factor V- I and a Factor VIII- I complex exists in plasma. If this were true, it would be conceivable that thrombin could bring Factors V and VIII into a reactive form by converting the fibrinogen into fibrin and subsequently breaking up the complex. Factors V and VIII have still more properties in common; both are absorbable to hydrophobic surfaces[61] and both are consumed during coagulation[61]. As will be shown below, the analogies between Factors V and VIII on the one hand and Factors X and IX on the other, were recently found in our laboratory to be even more striking than had previously been suspected. These analogies seem to be broad enough to justify the postulation of a new coagulation scheme.

Not much is known about the way in which Factor VIII is inactivated, although the process has some interesting and uncommon features[54].

Now, every case history of a severe haemophiliac shows that the absence of Factor VIII very severely hinders adequate thrombin formation; and, according to the above-mentioned studies of RAPPAPORT, thrombin is necessary for Factor VIII activation. This leaves us with the question: without either hen or egg, where does the genus gallus come from? A possible solution is found in the work of ALTMAN and HEMKER[1], who found that Factor XII and Factor XI can activate Factor VII just as tissue factor can. Therefore,

the sequence of Factors XII- XI- VII- X- V- II* can generate thrombin in the absence of tissue factor, but it does so very slowly. The small amount of thrombin formed by this pathway may be of no recognizable importance quantitativily, but may be very important indeed qualitatively, because it opens up the intrinsic pathway by its action on Factor VIII. Small amounts of thrombin may also be important by reason of their action on Factor V[38] and they are certainly important for their action on the blood-platelets[29]. The latter action, however, will not be reviewed here because blood-clotting pathways are complicated enough without the introduction of discussions on the complete mechanism of haemostasis. It needs only be mentioned that thrombin induces the liberation of phospholipids from the platelets.

It should be kept in mind that any overshooting of the coagulation process rapidly becomes fatal. Thrombosis and embolism constitute the highest contribution to death rates in Western society[16], most often by impairing the blood supply to the heart muscle. One of the mechanisms by which excessive activity of the coagulation process is prevented is illustrated by the reactions involving Factor VIII. Factor $VIII_t$ exists only as a relatively short-lived substance whose procoagulant power rapidly vanishes once it has been evoked. This mechanism is also found for Factor V and Factor II and it may exist for the Factors XI, X and VII as well[11]. There is also a possibility that the very action of a procoagulant (i.e. an activated clotting factor) triggers the generation of the activities that will subsequently inactivate this procoagulant. Factor V, at least, has been shown to be broken down by thrombin[56] under certain conditions. As thrombin is an activator of Factor V, we find the same difficulty here as we encountered with Factor VIII: How can Factor V be activated to form a prothrombin-converting enzyme, if thrombin is needed to make Factor V active?

ESNOUF[12] has found one answer to this question: Factor V does not seem to be compulsory for thrombin formation; it acts only as an accelerator. In our laboratory we have found another answer: Not only thrombin but also activated Factor VII may be able to activate Factor V[23].

*The factual correctness of the factor sequence X-V-II will be discussed below.

A crucial problem of the blood coagulation mechanism requires discussion now: What is the nature of the enzyme that converts prothrombin?

First, some words about the nomenclature. As long as the real nature of this enzyme is unknown, it is best denoted by the name of its substrate with the suffix -ase. Therefore, we shall call it prothrombinase or Factor II-ase. Formerly it was called "plasma-thromboplastin", especially when generated via the intrinsic pathway, but this term is confusing, because in everyday use it abbreviates to "thromboplastin" and then no distinction is possible with respect to tissue-thromboplastin, which is the name currently used for the mixture of tissue-factor and phospholipids that serves to activate Factor VII and thus to ensure the activity of the extrinsic pathway.

In the classic cascade schemes, prothrombinase is thought to be activated Factor V. MACFARLANE comes to this postulate by analogy from the other steps, and states that he cannot yet give experimental evidence for this view[35].

The school of RATNOFF has produced such experimental evidence[5,6], although other groups have put forward a considerable body of evidence that prothrombinase is not constituted from Factor V alone but from a complex comprising Factors V and X_a, Ca^{++}, and phospholipid. At the moment, the experimental results of RATNOFF *et al.* can be explained in terms of this alternative scheme, provided that the activation of Factor V by either activated Factor VII *or* thrombin is assumed. A great deal of experimental evidence appears to support the hypotheses that prothrombinase is a complex structure consisting of activated factor X and activated Factor V bound to a phospholipid surface, the former by a bond via a Ca^{++} ion, the latter by a hydrophobic bond. This concept is shown in Fig. 4.

The most compelling points on which this view is based are:

(a) Anyone of the three components, Factor X_a, Factor V_a, or phospholipid, can be present in a limiting amount. The concentration of prothrombinase is then determined by the concentration of this limiting component[13].
(b) An excess of phospholipid has an inhibitory action, bly presuma because this excess results

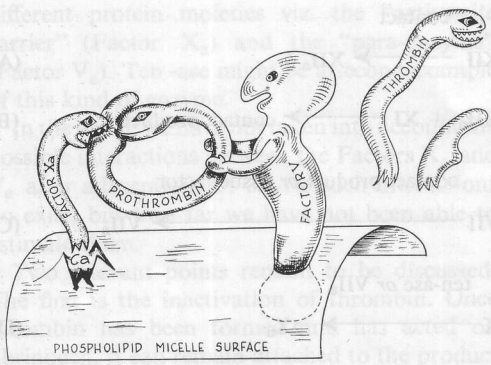

Fig. 4. The happening by which thrombin is generated.

in a surface area too large for Factors X and V to cover adequately. A possible explanation of the strongly inhibitory action of certain specific phospholipids[28] could lie in the fact that these phospholipids bind only one of the two factors and thus withdraw a reactant from the mixture.

(c) From a mixture having prothrombinase activity, no Factor X, Factor V, or phospholipids can be separated without loss of that activity[13].

(d) Enzyme kinetics of one-stage estimations of Factors II, V, X, and phospholipid show that the Factor II- converting enzyme must be a combined product of Factors X, V, and phospholipid, and also that this product must be a labile one that is actively degraded during the clotting process. This is another indication of the importance of the inactivating processes during clotting[25].

(e) Experiments on the generation of prothrombinase from purified bovine Factor V, Factor X, and phospholipid show that the kinetics involved are compatible with the concept of prothrombinase as a combination product of the reactants but not with the concept implying the activation of Factor V by Factor X_a[20]. Therefore the results of these experiments support those mentioned under d. Moreover, it has been shown that in a purified system prothrombinase is about 10 times more stable than it is in plasma, which supports the assumption of a prothrombinase inactivator in plasma.

(f) Activated Factor X can be shown to attach to phospholipid when Ca^{++} is present. Factor V binds to phospholipid in the absence of Ca^{++}. Excess Ca^{++} prevents the binding of Factor V to phospholipid[51,52,53] (Table II).

Activated Factor X has been shown by MACFARLANE[34] and ESNOUF and WILLIAMS[15] to originate from Factor X by the action of RUSSEL's Viper Venom or Factor VII_a. HANAHAN[41,18] has shown that one molecule of Factor X gives rise to two molecules of Factor X_a when Ca^{++}ions are present.

Recent experiments in our laboratory, performed in collaboration with M. J. P. KAHN, have shown that Factor IX_a, like Factor X_a, is bound to phospholipid with the aid of Ca^{++}ions (Table II, ref. 22) and that Factor VIII, like Factor V, is bound to phospholipid via a kind of binding that is hampered by an excess of Ca^{++}ions.

It has long been known that phospholipid and Ca^{++} play a role in coagulation at the level of Factors VIII and IX as well as at the level of

TABLE II: ADSORPTION OF CLOTTING FACTORS ONTO PHOSPHOLIPID UNDER INFLUENCE OF Ca^{++}IONS*

Ca^{++} concentrates (mM)	Percentage adsorbed of Factor V	Factor VIII	Factor IX	Factor X
1	65	67	23	37
50	2	41	45	59
100	<1	3	71	77

* Serum was the source of Factor IX and Factor X; $BaSO_4$-adsorbed plasma was the source of Factor V and Factor VIII. The phospholipid used was Inostithin (Asc. Concentrates Ltd.). For further experimental details see ref. 62.

Factors V and X. Now that the strikingly similar mode of interaction of these two pairs of factors with phospholipid and Ca^{++} has been observed and a reasonable assumption can be made concerning the exact mode of interaction between Factors V and X, phospholipid, and Ca^{++}, it requires only a little imagination to postulate that a complex with the enzymatic activity to convert Factor X into its acitvated form is constituted by Factor IX_a and Factor VIII together, adsorbed onto a phospholipid micelle surface. Because of its enzymatic activity, the complex is termed ten-ase in the reaction equations below.

From the foregoing a general pattern of the interaction of clotting factors emerges in which the enzymatic activity is exerted by clusters of two factors adsorbed onto phospholipid rather than by activated clotting factors alone.

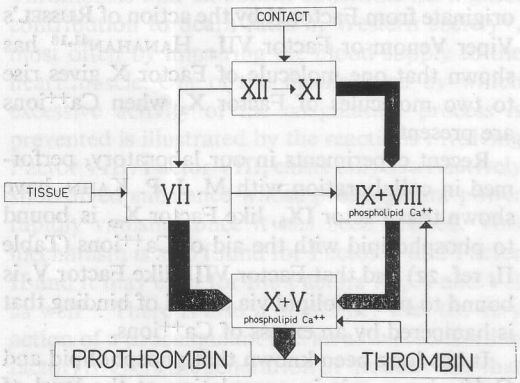

Fig. 5. A clotting scheme anno 1967. In this diagram the arrows indicate "action upon" rather then "convertion into"; the thickness of the arrows indicates their relative importance.

An over-all scheme based on this principle is given in Fig. 5. This figure also indicates the role conceivably played by Factor VII in the intrinsic pathway. The horizontal arrow between Factor VII and Factor IX refers to a finding by Josso and PROU-WARTELLE[27], indicating that under certain circumstances Factor VII_a may act on Factor IX.

Written in reaction equations, the process looks like this:

contact

$$XII \longrightarrow XII_a \quad (A)$$

$$XII_a + XI \longrightarrow \text{contact product} \quad (B)$$

contact product *or* tissue factor

$$VII \longrightarrow VII_a \quad (C)$$

ten-ase *or* VII_a

$$X \longrightarrow 2X_a \quad (D)$$

X_a *or* VII_a *or* thrombin

$$V \longrightarrow V_a \quad (E)$$

$$X_a + V_a + \text{ph.lip.} + Ca^{++} \longrightarrow \text{prothrombinase} \quad (F)$$

prothrombinase

$$II \longrightarrow \text{thrombin} \quad (G)$$

contact product

$$IX \longrightarrow IX_a \quad (H)$$

thrombin

$$VIII \longrightarrow VIII_a \quad (I)$$

$$IX_a + VIII_a + \text{ph.lip.} + Ca^{++} \longrightarrow \text{ten-ase} \quad (K)$$

It is interesting to speculate upon the role of Factor V in the prothrombinase complex. Experiments reported by ESNOUF[12] indicate that the active center of the prothrombinase complex is located on the Factor X_a molecule. This is the more feasible because Factor X_a appears to be an esterase of the serine-esterase family whereas of Factor V_a no such properties could be found. This suggests that Factor V_a might act by providing means to achieve a more efficient handling of the substrate (=prothrombin) by the active center. When this is true, it would, to our knowledge, be the first recorded instance of an enzyme (prothrombinase) consisting of two

different protein moieties viz. the "active-site carrier" (Factor X_a) and the "para-enzyme" (Factor V_a). Ten -ase might be a second example of this kind of enzyme.

In the above we have not taken into account the possible interactions between the Factors X_a and V_a after adsorption. Of course such interactions do exist, but thus far we have not been able to estimate them.

Two relevant points remain to be discussed. The first is the inactivation of thrombin. Once thrombin has been formed and has acted on fibrinogen, it can remain attached to the product molecule during polymerization[50]. Fibrin has a wel-defined thrombin-absorbing action. Moreover, free thrombin in plasma is inactivated in a reaction with a plasma protein known as antithrombin III. The affinity of thrombin for antithrombin III is, however, considerably smaller than the affinity of thrombin for fibrinogen, so that without hindering the conversion of fibrinogen, antithrombin III can remove free thrombin from clotting blood[26]. Partially antithrombin III- deficient patients have been described[11]. These patients develop severe thrombotic complications at the slightest provocation.

The second point is that the product formed by polymerization of fibrin monomers is not a firm clot; unlike a normal clot, for instance, it is soluble in 5 M urea. In the polymerizate, Factor $XIII_a$, the fibrin-stabilizing factor, constructs bridges between adjacent amino acid chains. Factor $XIII_a$ originates from Factor XIII by the action of thrombin. The bridging is brought about by catalization of an interaction between amino group containing amino acid residues in parallel fibrin strands[32]. (Fig. 6, 7).

In conclusion it may be said that blood coagulation is an extremely complicated process. The complications have a magic charm for the specialized investigator and often appear as uncanny black magic to the outsider, who may derive only a limited amount of comfort from the fact that of late much of the confusion has been replaced by recognized complexity, even more limited because the recent views and hypotheses discussed in this article are liable to be corrected and extended over and over again in the future. The only real comfort for the layman and specialist alike may be that "Truth comes out of error more readily than out of confusion" (FRANCIS BACON).

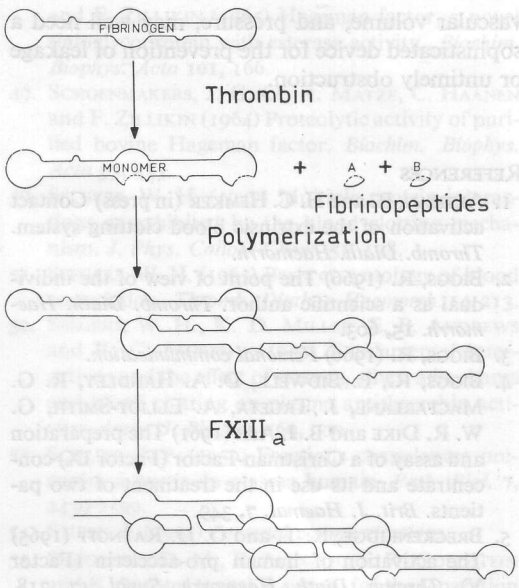

Fig. 6. Schematic representation of the polymerization of fibrinogen.

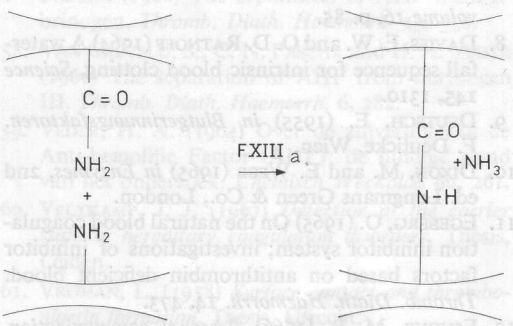

Fig. 7. The action of the fibrin stabilizing factor (Factor XIII).

Those who object intuitively to the idea of so complicated a mechanism for so simple a function as blood clotting are reminded that circulation, which is well known to be provided with a most intricate mechanism for maintaining flow, intra-

vascular volume, and pressure, may well need a sophisticated device for the prevention of leakage or untimely obstruction.

References

1. Altman, R. and H. C. Hemker (in press) Contact activation of the extrinsic blood clotting system. *Thromb. Diath. Haemorrh.*
2. Biggs, R. (1966) The point of view of the individual as a scientific author. *Thromb. Diath. Haemorrh.* **15,** 603.
3. Biggs, R. (1966) *Personal communication.*
4. Biggs, R., E. Bidwell, D. A. Handley, R. G. Macfarlane, J. Trueta, A. Elliot-Smith, G. W. R. Dike and B. J. Ash (1961) The preparation and assay of a Christman-Factor (Factor IX) concentrate and its use in the treatment of two patients. *Brit. J. Haemat.* **7,** 349.
5. Breckenridge, R. T. and O. D. Ratnoff (1965) The activation of human pro-accelerin (Factor V). *Thromb. Diath. Haemorrh. Suppl. 17,* 218.
6. Breckenridge, R. T. and O. D. Ratnoff (1966) The activation of pro-accelerin by bovine thrombokinase (with notes on the antithrombic activity of phenylmethylsulfonylfluoride). *Blood* **27,** 527.
7. Cunningham, L. (1965) The structure and mechanism of action of proteolytic enzymes, *in* M. Florkin and E. H. Stotz, *Comprehensive Biochemistry* 1st. ed., *Elsevier,* Amsterdam-London-New York, *volume 16,* p. 85.
8. Davies, E. W. and O. D. Ratnoff (1964) A waterfall sequence for intrinsic blood clotting, *Science* **145,** 1310.
9. Deutsch, E. (1955) *in Blutgerinnungsfaktoren.* F. Deuticke, Wien.
10. Dixon, M. and E. Webb (1965) *in Enzymes,* 2nd ed. Longmans Green & Co., London.
11. Egeberg, O. (1965) On the natural blood coagulation inhibitor system; investigations of inhibitor factors based on antithrombin deficient blood. *Thromb. Diath. Haemorrh.* **14,** 473.
12. Esnouf, M. P. (1966) *Personal communication.*
13. Esnouf, M. P. and F. Jobin (1965) Lipids in prothrombin conversion. *Thromb. Diath. Haemorrh. Suppl. 17,* 103.
14. Esnouf, M. P., F. Jobin and J. C. Peden (1963) Purification of bovine Factor V. *Biochem. J.* **89,** 44.
15. Esnouf, M. P. and W. J. Williams (1962) The isolation and purification of a bovine-plasma protein which is a substrate for the coagulation fraction of Russells Viper Venom. *Biochem. J.* **84,** 62.
16. Fejfar, Z., D. Badger and M. Crais (1966) Epidemiological aspects of thrombosis and vascular disease. *Thromb. Diath. Haemorrh. Suppl. 21,* 5.
17. Florkin, M. and E. H. Stotz (1965) *in* M. Florkin and E. H. Stotz, *Comprehensive Biochemistry, Volume 13,* 2nd ed., Elsevier, Amsterdam-London New York.
18. Hanahan, D. J. and D. Papahadjopoulos (1965) Interactions of phospholipids with coagulation factors. *Thromb. Diath. Haemorrh. Suppl. 17,* 71.
19. Hemker, H. C. (1965) *Kinetic properties of an enzyme cascade.* In discussion to R. G. Macfarlane, see ref. 36 *Thromb. Diath. Haemorrh. Suppl. 17,* 63.
20. Hemker, H. C., M. P. Esnouf, P. W. Hemker, A. C. W. Swart and R. G. Macfarlane (in press) Kinetic aspects of the interaction of purified blood clotting factors. *Nature.*
21. Hemker, H. C., N. Fekkes, A. Hensen, H. Schrijver and E. A. Loeliger (1966) Quantitation of circulating fibrinogen breakdown products in intravascular clotting. *Thromb. Diath. Haemorrh. Suppl. 22,* 227.
22. Hemker, H. C. and M. J. P. Kahn (in press) The reaction sequence of blood coagulation. *Nature.*
23. Hemker, H. C. and M. J. P. Kahn (1967) *Personal communication.*
24. Hemker, H. C., D. Ménaché and J. P. Soulier (1964) Specific activity of prothrombin. *Proc. Europ. Fed. Biochem. Soc.* London A 62, p. 48.
25. Hemker, H. C. and J. M. C. Wimmers (1965) On the reaction mechanism of the extrinsic blood clotting system. *Proc. Europ. Fed. Biochem. Soc.* Vienna A 120, p. 82.
26. Hensen, A and E. A. Loeliger (1963) Antithrombin III. *Thromb. Diath. Haemorrh. Suppl. 10.*
27. Josso, F. and O. Prou-Wartelle (1965) Interaction of tissue-factor and Factor VII at the earliest phase of coagulation. *Thromb. Diath. Haemorrh. Suppl. 17,* 35.
28. Kahn, M. J. P., M. Hans and R. H. Bourgain (1965) The action on blood clotting of different lipoid fractions of Beaf brain. (Methods of fractionization and chromatographic isolation procedure of a phospholipid anticoagulant). *Thromb. Diath. Haemorrh.* **14,** 445.
29. Käser-Glanzmann, R. and E. F. Lüscher (1962) The mechanism of platelet aggregation in relation to hemostasis. *Thromb. Diath. Haemorrh.* **7,** 480.
30. Lechner, K. (1965) On the source of autoprothrombin C. *Thromb. Diath. Haemorrh. Suppl. 17,* 259.
31. Levine, S. N. (1966) Enzyme amplifier kinetics. *Science* **152,** 651.
32. Loewy, A. G. (1964) Fibrinase (Factor XIII). *Thromb. Diath. Haemorrh. Suppl. 13,* 109.
33. Lukosevicius Adams, A. and L. Vroman (1965)

Observation of Factor XII and Factor XII deficient plasma at an interface. *Thromb. Diath. Haemorrh.* **13,** 387.

34. MACFARLANE, R. G. (1961) The coagulant action of Russells Viper Venom. The use of antivenom in defining its reaction with a serum factor. *Brit. J. Haemat.* **7,** 498.
35. MACFARLANE, R. G. (1964) An enzyme cascade in blood clotting mechanisms, and its function as a biochemical amplifier. *Nature* **202,** 498.
36. MACFARLANE, R. G. (1965) A clotting scheme for 1964. *Thromb. Diath. Haemorrh. Suppl. 17,* 45.
37. MACFARLANE, R. G. (1966) The basis of the cascade hypothesis in blood clotting. *Thromb. Diath. Haemorrh.* **15,** 591.
38. NEWCOMB, T. F. and M. HOSHIDA, (1965) Factor V and thrombin. *Scand. J. Clin. Lab. Invest.* **17,** *suppl. 84,* 61.
39. NIEWIAROWSKI, S., J. STAGURSKA and Z. WEGRZYNOWICS (1962) Arginine esterase activity of the contact factor. *Thromb. Diath. Haemorrh.* **7,** 514.
40. PAPAHADJOPOULOS, D. and D. J. HANAHAN (1964) Observations on the interactions of phospholipids and certain clotting factors in prothrombin activator formation. *Biochim. Biophys. Acta* **90,** 436.
41. PAPAHADJOPOULOS, D., C. HOUGIE and D. J. HANAHAN (1964) Purification and properties of bovine Factor V. *Biochemistry* **3,** 264.
42. PAPAHADJOPOULOS, D. C. HOUGIE and E. T. YIN (1964) Purification and properties of bovine Factor X: molecular changes during activation. *Biochemistry* **3,** 1931.
43. PASSAGLIA, E., R. R. STROMBERG and J. KRUGER edts. (1964) *Ellipsometry in the measurement of surfaces and thin films.* National Bureau of Standards Miscellaneous Publication 256, Washington D.C.
44. PASCHA, C. N. (1963) *Een bijdrage tot de zuivering van de antihemofiliefactor.* Thesis, Amsterdam.
45. PRYDZ, H. (1965) Some characteristics of purified Factor VII preparations. *Scand. J. Clin. Lab. Invest.* **17,** *suppl. 84,* 78.
46. RAPAPORT, S. I., P. F. HJORT and M. J. PATCH (1965) Further evidence that thrombin-activation of Factor VIII is an essential step in intrinsic clotting. *Scand. J. Clin. Lab. Invest.* **17,** *suppl. 84,* 88.
47. SCHOENMAKERS, J. G. G. (1965) *De Hageman factor.* Thesis, Nijmegen.
48. SCHOENMAKERS, J. G. G., R. MATZE, C. HAANEN and F. ZILLIKIN (1965) Hageman factor, a novel sialoglycoprotein with esterase activity. *Biochim. Biophys. Acta* **101,** 166.
49. SCHOENMAKERS, J. G. G., R. MATZE, C. HAANEN and F. ZILLIKIN (1964) Proteolytic activity of purified bovine Hageman factor. *Biochim. Biophys. Acta* **93,** 433.
50. SEEGERS, W. H. (1947) Multiple protein interactions as exhibited by the blood clotting mechanism. *J. Phys. Colloid Chem.* **51,** 198.
51. SEEGERS, W. H. (1965) Basic enzymology of blood coagulation. *Thromb. Diathes. Haemorrh.* **14,** 213.
52. SEEGERS, W. H., K. D. MILLER, E. B. ANDREWS and R. C. MURPHY (1952) Fundamental interactions and the effect of storage, ether, adsorbants and blood clotting on plasma antithrombin activity. *Amer. J. Physiol.* **169,** 700.
53. SOULIER, J. P. (1959) Fractions coagulantes préparées à partir du plasma humain. *Path. Biol.* **7,** 2439-2599.
54. STIBBE, J. (1964) *Personal communications.*
55. SURGENOR, D. M., B. ALEXANDER, R. GOLDSTEIN and K. SCHMID (1955) A system for the separation of the protein components of human plasma: II-the components of the clotting process. *J. Phys. Colloid. Chem.* **55,** 94.
56. SURGENOR, D. M., N. A. WILSON and A. S. HENRY (1961) Factor V from human plasma. *Thromb. Diath. Haemorrh.* **5,** 1.
57. VAN CREVELD, S., I. A. MOCHTAR and C. N. PASCHA (1966) The separation of AHF from fibrinogen. *Thromb. Diath. Haemorrh.* **15,** 338.
58. VAN CREVELD, S., C. N. PASCHA and H. A. VEDER (1961) The separation of AHF from fibrinogen III. *Thromb. Diath. Haemorrh.* **6,** 282.
59. VEDER, H. A. (1964) Over de zuivering van de Anti-hemofilie Factor (AHF); de huidige stand van het onderzoek. *Chemisch Weekblad* **60,** 261.
60. VELTKAMP, J. J. (1967) *Detection of the carrier state in hereditary coagulation disorders.* Thesis, Leiden.
61. VROMAN, L. (1958) *Surface contact and thromboplastin formation.* Thesis, Utrecht.
62. VROMAN, L. (1964) Effects of hydrophobic surfaces upon blood coagulation. *Thromb. Diath. Haemorrh.* **10,** 445.
63. WIMMERS, J. M. C. and H. C. HEMKER (1966) *Scheiding van het prothrombine complex.* In Pr. 7de Fed. Verg. Med. Biol. Vereenigingen p. 131. Amsterdam.